

AN EVALUATION OF THE RELATIONSHIP BETWEEN SIGMA-1 AND SIGMA-2
RECEPTORS AND THE ENDOCANNABINOID SYSTEM

by

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the requirements of the Sally McDonnell Barksdale Honors College.

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First, I would like to take this opportunity to dedicate this thesis to my family. Without my parents' constant support, encouragement, and dedication to my education, I would not be where I am today. It is their hard work that has allowed me to be a member of the University of Mississippi Sally McDonnell Barksdale Honors College and participate in this research project. I would also like to dedicate my thesis to my siblings and my friends, who are always ready to lend a listening ear, take a weekend to visit me, or offer sound advice. Your support means everything!

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ABSTRACT

JENN MILLER: An Evaluation of the Relationship between Sigma-1 and Sigma-2 Receptors and the Endocannabinoid System
(Under the direction of Lisa Wilson and Dr. Christopher McCurdy)

Within the past two decades, sigma receptors have become a popular area for research. Although much has been learned about their structure, subtypes, and functions; there is still much to be learned. Consisting of two receptor subtypes (sigma-1 and sigma-2), it has been discovered that sigma receptor ligands potentiate the analgesic effects of both opiates and cannabinoids, though the exact mechanism and sigma subtype on which this occurs is still unknown. The purpose of this study is to determine if potentiation of opiates and cannabinoids occurs through sigma-1 or sigma-2 receptor signaling. Tetrad assays were performed for:

1. Morphine, an opiate, at multiple doses (i.p.),
2. CP 55,940, a cannabinoid, at multiple doses (i.p.),
3. CM304, a sigma-1 antagonist, at multiple doses (i.p.), and
4. CM398, a sigma-2 antagonist, at multiple doses (i.p.).

The potentiation studies were then completed by using both 20 and 45 mg/kg doses of CM304 and CM398 against either a 1 mg/kg dose of CP 55,950 or a 2 mg/kg dose of morphine. The CM dose was administered 15 minutes before the CP or the morphine dose, and the analgesic study was performed 15 minutes post CP or morphine administration. This study revealed that CM304, the sigma-1 antagonist, potentiated the effects of CP 55,940 and morphine for the hotplate assay while it attenuated the effects

on the tail-flick assay. Additionally, CM398, the sigma-2 antagonist, failed to potentiate both CP 55,940 and morphine for both the hotplate and the tail-flick assay. Results also showed that AZ66, the general sigma receptor antagonist, potentiated the effects of CP 55,940 for both the hotplate and the tail-flick assays. In conclusion, administering a sigma-1 antagonist, instead of a sigma-2 antagonist, in conjunction with either an opiate or a cannabinoid will potentiate the effects of the challenge drug. This can serve as an important basis for the future of pain research.

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I. Background

The sigma receptor was initially categorized as another opioid receptor subtype (Maurice et al., 2009). The confusion arose because the ligands that were used in the experiment cross-reacted with both sigma receptors and opioid receptors (Maurice et al., 2009). Further studies demonstrated that the sigma receptor was a separate receptor from the opioid receptor. The sigma receptor is a unique chaperone protein found mainly in the endoplasmic reticulum and the plasma membrane of cells. In these cells, there are two known subtypes of the sigma receptor: sigma-1 and sigma-2 (Maurice et al., 2009).

Sigma-1 was the first sigma subtype to be discovered and in 1996, it became the first subtype to be cloned from a guinea pig liver (Zamanillo et al., 2013). It was subsequently cloned from mouse, rat, and human (Hanner et al., 1996; Mei et al., 2001; Pan et al., 1998; Seth et al., 1997; Seth et al., 1998). The sigma-1 gene encodes a 25-29 kDa molecular mass protein that consists of 223 amino acids and at least one transmembrane spanning domain (Zamanillo et al., 2013; Ayudar et al., 2002; Jbilo et al., 1997). It has been found to be broadly distributed in both the peripheral organs and the central nervous system, including high expression in the brain, the heart, the liver, the spleen, and the GI tract (Matsomoto et al., 2007). More specifically, the sigma-1 receptor has been localized to regions of the brain associated with pain control, including the superficial layers of the dorsal horn, the periaqueductal gray matter, the locus coeruleus, and the rostroventral medulla (Zamanillo et al., 2013). Because the sigma-1 subtype has been sequenced, cloned, and is the most well-understood sigma subtype, it has been used as the basis for many studies regarding disease states. Currently, sigma-1 is hypothesized to play a very important role in addiction, pain, depression, Alzheimer's disease,

schizophrenia, stroke, HIV, cancer, and many other neurological conditions (Maurice et al., 2009). Sigma-1 subtype receptors will continue to be researched extensively as a potential treatment option for many of the aforementioned conditions.

Despite the sigma-2 receptor being the only other sigma subtype, there has not been a large amount of research performed about its roles and its capabilities. This lack of research has led to some confusion surrounding the sigma-2 gene and its corresponding protein. Although the Progesterone Receptor Membrane Component 1 (PGRMC1) has been recently implicated as a sigma-2 subtype, subsequent research now indicates that this might not be the case (Chu et al., 2015). From this study, data indicates that PGRMC1 and sigma-2 receptors are genetically different, meaning that they are two different proteins and that PGRMC1 is a non-sigma-2 receptor binding site in mammalian tissues (Chu et al., 2015). More basically, however, there is still some literature on the structure and the proposed function of this subtype. Sigma-2 is slightly smaller in size than sigma-1, existing as an 18-22 kDa protein that is highly expressed in the brain, the liver, and the GI tract (Matsomoto et al., 10). Additionally, sigma-2 is different because it is infrequently expressed in the heart and the spleen (Matsomoto et al., 10). The functions of sigma-2 are also believed to be vastly different from sigma-1. Thus far, sigma-2 has been linked to roles in cellular events, such as proliferation, apoptosis, dendritogenesis, synaptogenesis, neuronal plasticity, activation of cytochrome P450, and steroid signaling (Zamanillo et al., 2013). It has also been speculated that the binding of sigma-2 ligands to sigma-2 receptors can trigger both caspase-dependent and caspase-independent apoptosis (Zeng et al., 2014). More research is still needed to fully understand these processes with regards to sigma-2.

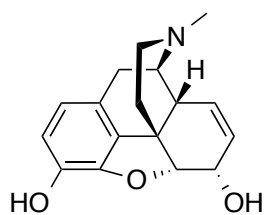
The initial confusion surrounding the classification of sigma receptors as opioid receptors led researchers to investigate the relationship between opioids and sigma receptors. Although research reveals a clear relationship between the two, it is still unknown whether or not the sigma receptors interact directly with opioid receptors or alter signaling pathways downstream from the opioids (Matsomoto et al., 2007). In fact, it has been shown that sigma-1 antagonists can potentiate the effects of opioid analgesia which will be detailed later. Regardless of the many questions surrounding the specifics of the relationship, there is still a multitude of literature that describes opioid analgesia and sigma receptors (Sánchez-Fernández et al., 2014; Vidal-Torres et al., 2013; Tseng et al., 2011; Kim et al., 2010; Mei et al., 2007; Mei et al., 2002).

Although moderate to severe pain is a very common medical complaint among patients, it is still a very complicated condition to manage. In most communities today, pain is managed by giving opioids, like morphine, to patients. Although opioids have strong analgesic effects, they can also produce many harmful side effects, including constipation, nausea, respiratory distress, tolerance, and addiction liability (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Because of these side effects, researchers and clinicians are investigating new ways to manage pain, particularly by giving opioids in conjunction with other drugs (Lui et al., 2011; Khan et al., 2011). The goal of giving an additional drug, like a sigma antagonist, is to enhance the effects of the opioid without also increasing the side effects. One example that has demonstrated efficacy is the use of sigma-1 antagonists. Additionally, sigma receptor antagonists alone are believed to play a potential key role in the management of pain.

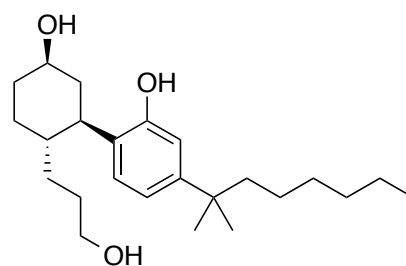
In previous studies with mice, it was shown that by giving an intrathecal injection of a sigma-1 receptor antagonist in conjunction with an opioid, the opioid-induced analgesia was potentiated (Maurice et al., 2009). Instead, if a sigma-1 agonist was administered with the opioid, then the opioid-induced analgesia was attenuated (Maurice et al., 2009). In other words, the antagonist enhanced the pain-relieving effects of the opioid while the agonist diminished the pain-relieving effects of the opioid. In similar studies, the sigma-1 receptors were down-regulated and then knocked-out completely to see if the same effects could be observed. When sigma-1 receptors were down-regulated, the analgesic effects of the opioid were again potentiated (Maurice et al., 2009). However, when sigma-1 receptors were knocked-out entirely, the analgesic effects of the opioid were not potentiated (Zamanillo et al., 2013). This reveals that sigma-1 receptors have some type of modulating capabilities over opioid analgesia, which is still not fully understood. Perhaps the best result of these studies is the evidence that shows that the side effects, like tolerance, withdrawal symptoms, and constipation were not also potentiated (Vidal-Torres et al., 2013). While the analgesic effects of opioids were able to be potentiated, the side effects for that specific dose remained the same. This means the pain-relieving effects were enhanced while the side effects were not. This looks very promising for future treatment and management of pain. Lastly, there has not been much research, if any at all, for the role of sigma-2 in the treatment and management of pain.

To further examine the role of sigma-1 and sigma-2 receptors in the management of pain, a number of compounds were used for this research. Morphine, an opioid, was used to show effects that have already been described in previous literature (Structure 1). Although the opioid system and the endocannabinoid systems are very similar, there are

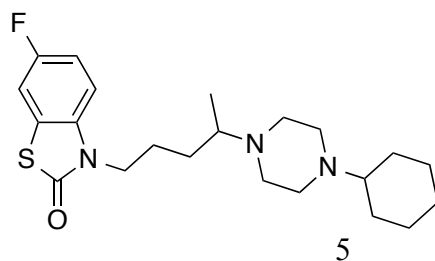
no literature reports that have investigated an interaction between the sigma receptors and the endocannabinoid receptors. However, research performed in our lab revealed that the administration of a sigma antagonist with a cannabinoid also produced potentiation of analgesic effects (unpublished results). Because of this previous research, we included a cannabinoid in the experiment. In this experiment, CP55,940 was used as the cannabinoid. CP55,940 is a synthetically cannabinoid that mimics the properties of naturally-occurring Δ^9 THC (Structure 2) (Wilson et al., 2016). It is a full agonist to cannabinoid-1 receptors (CB₁) and is up to ten times more potent than Δ^9 THC. To test the potentiation effects on sigma-1 and sigma-2 receptors, three antagonists were used. AZ66 is a compound that is prepared and synthesized in Dr. McCurdy's lab at the University of Mississippi (Structure 3). It has high binding affinity for both sigma-1 and sigma-2 receptors and a >200-fold preference for sigma receptors than any other site tested in its original synthesis and testing research (Seminario et al., 2011). In addition to the general sigma antagonist, more specific subtype antagonists were also used. CM304 served as the sigma-1 antagonist (Structure 4) and CM398 as the sigma-2 antagonist. Both are synthetically-made antagonists (Structure 5).



Structure 1: Morphine

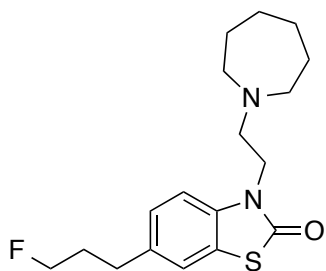


Structure 2: CP 55,940

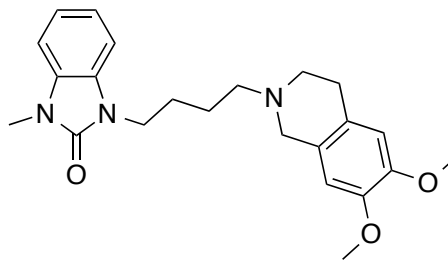


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Structure 3: AZ66



Structure 4: CM304



Structure 5: CM398

Because of the abundance of research surrounding analgesia and sigma-1 and the lack of research surrounding analgesia and sigma-2, the aim of this research is to investigate the potentiation effects of opiates and cannabinoids on both sigma-1 and sigma-2 subtypes. The goal is to determine if potentiation is greater for opioids with sigma-1 antagonists or opioids with sigma-2 antagonists. Similarly, it is also to determine if potentiation is greater for cannabinoids with sigma-1 antagonists or cannabinoids with sigma-2 antagonists. The potential significance of these results suggest that it can be possible to administer a sigma-1 antagonist as an adjuvant to a cannabinoid agonist in order to enhance the analgesic effects while minimizing the development of side effects due to lower doses being utilized. This could result in potentially decreasing the cannabinoids associated tolerance and addiction liability while effective analgesic can be maintained.

II. Methods

Subjects

Adult male black C57BL6 mice (18-32g) were obtained from Harlan Laboratories and used for all of the tests. All animals were housed five to a cage and received food/water ad lib. The housing facilities were maintained on a 12 hour light/dark schedule (lights on at 6:00am and off at 6:00pm). CP 55,940 was acquired from Tocris

Bioscience (Bristol, United Kingdom). Morphine, Cremophor and Ethanol were obtained from Sigma Aldrich (Bellefonte, PA). Lastly, CM304, CM398, and AZ66 were all prepared and synthesized in Dr. McCurdy's lab as a part of the Department of BioMolecular Sciences Division of Medicinal Chemistry. All methods performed were approved by the Institutional Animal Care and Use Committee (IACUC).

Drug Preparation

All drugs were dissolved according to the methods of Olson et al (1973). A mixture of Ethanol, Cremophor, and Saline was prepared using a ratio of (1:1:18). Drugs were completely dissolved into ethanol before adding Cremophor and saline. Drugs were delivered to the animals using an intraperitoneal (i.p.) injection (Wilson et al., 2016).

Tetrad Assay

The mouse tetrad is a behavioral assay developed to characterize the biological effects of cannabinoids and opiates using locomotor activity, nociception, changes in body temperature, and catalepsy (Little et al., 1988). The assay has been well documented to indicate that the typical effects of cannabinoids is decreased locomotion, increased cataleptic activity, increased antinociception, and hypothermia (Pertwee et al., 2008). Twenty-four hours prior to the start of the experiment the mice were acclimated for 15 minute increments to the cold hotplate container and apparatus. On the experimental day, the mice were brought into the experimental room and allowed to acclimate to the room settings for 30 minutes and then to the locomotor chamber for 30 minutes (Wilson et al., 2016). Once the second thirty minute acclimation period was over, baseline readings for supraspinal antinociception (hotplate), catalepsy, hypothermia, and spinal antinociception (tail-flick) were recorded pre-injection.

In the hotplate assay, the subject was placed on a hotplate at 52°C inside of a plastic cylinder, so that the subject was contained in one area. The timer was manually started and then stopped once one of the cues was performed by the subject. These cues included licking the back paw, moving the back paw to the side surface of the cylinder, jumping, and rapidly tapping one of the back paws. Because mice lick their front paws during grooming, only the activity of the back paws is marked as perception of pain. Additionally, the cut-off time for this assay is 45 seconds to reduce the possibility of tissue damage to the subject. The purpose of the hotplate is to measure the subject's perceived pain and the perceived peripheral pain analgesic effects of the drugs.

With the purpose of measuring the psychoactive effects of the drug, the catalepsy test is the second test included in the tetrad. In this test, the subject's front paws are placed on a metal bar and his hind paws reside on the lower surface. Once the subject is in this initial position, the timer is started and continues until the subject either jumps onto the metal bar or lowers his front paws onto the lower surface. If the subject remains in the initial position for more than five seconds, then it is considered cataleptic and unaware of its surroundings. The cut-off time for this assay is three minutes. This method makes it easy to determine the psychoactive effects of the drug on the subject.

The third test of the tetrad is hypothermia, or a measurement of the core body temperature of the subject. The temperature is measured by inserting a temperature probe into the subject's rectum to note any changes in body temperature between pre- and post-drug injection.

In the last test of the tetrad, the spinal antinociception properties were recorded in the tail-flick assay. To gain these measurements, the subject was placed in a plastic

restrainer so that its tail was hanging out of the restrainer. The restrainer was then laid down on the surface of the machine to ensure that the tail was also flat along the surface of the machine. Once the subject had settled down into this position, the timer was started and a high-energy beam of light was projected on the distal portion of the subject's tail. Once the subject moved its tail out of the path of the light beam, the light and timer automatically shut off. This was repeated once more and the average of the two trials was taken to ensure greater accuracy. To minimize potential tissue damage, the cut-off time for this assay was 15 seconds. The purpose of the tail-flick assay is to measure the spinal (reflex) analgesic effects of the administered drug.

Once the baseline readings were obtained for the tetrad, the animals were injected i.p. with either the (1:1:18) vehicle, CP 55,940 (0.1, 0.25, 0.5mg/kg), morphine (2, 2.5, 5 mg/kg), CM304 (5, 10, 20 mg/kg), or CM398 (5, 10, 20 mg/kg). The animals were then allowed to move around in their individual locomotor chamber (San Diego Instruments) for 30 minutes. The locomotor chamber consists of a 16 x 16 beam ray that detected movement of the animal. Breaking the photobeams was then quantified as a measure of locomotor activity. The last 10 minutes of quantifying time was used for data analysis (Wilson et al., 2016). Evaluating locomotor activity provided an insight to the sedative effects of the drug. The larger the sedative effects of the drug, the fewer photobeams that were broken.

Thirty minutes post-injection the subject was removed from the locomotor chamber and again run through the tetrad assay. Hotplate latency, catalepsy, core body temperature, and tail-flick latency were recorded.

Potential Studies

The purpose of the potential studies is to measure the analgesic effects of the drug on the mice in the experiment. In these studies, only the hot plate assay and the tail-flick assay were used (the same procedures as the ones described above). Again, the analgesics of the mice were compared before and after the administration of the two drugs. Twenty-four hours before the testing the mice were acclimated for fifteen minutes to the hotplate surface. On the day of the experiment, the mice were brought into the lab and allowed to acclimate for thirty minutes. After this thirty minute period, baseline readings for supraspinal (hot plate) and spinal (tail-flick) nociception were measured. Then, the mice were injected i.p. with the antagonist drug, which was either CM304 (20, 40 mg/kg), CM398 (20, 40 mg/kg), or AZ66 (20 mg/kg). The mice were placed back in their cages for fifteen minutes, after which the mice were again injected i.p. with the challenge drug, either 2 mg/kg morphine or 0.1 CP 55,940. Fifteen minutes after the challenge drug injection, the hotplate and tail-flick latencies were recorded. The goal is to see whether or not CM304, CM398, and/or AZ66 potentiated the effects of CP 55,940 and morphine.

Data Analysis

Data was shown as mean \pm SEM. with each group having n=10 animals. Both hotplate and tail-flick were expressed as percent maximum effect (%MPE=[(post-drug latency-basal latency)/(cutoff latency-basal)]x 100 (Little et al., 1998). Statistical analysis was performed using one way ANOVA preceded by the Dunnett's post hoc test for locomotor activity and Tukey post hoc test for hot plate, cataleptic effects, decrease in

rectal temperature and tail-flick to define significant different against the vehicle control at $p < 0.05$ for each specific time point (Wilson et al., 2016).

III. Results

Tetrad Assays

I. Locomotor Activity

Figures 1 and 2 demonstrate the locomotor activity dose response curves for CM304 and CM398, respectively. Both graphs also illustrate the response when administered (i.p.) the vehicle (1:1:18) and a 2.5 mg/kg dose of CP 55,940. The 2.5 mg/kg dose of CP 55,940 with both CM304 and CM398 produced a p value < 0.001 . For CM304, 5 mg/kg generated a p value < 0.05 . Both 10 mg/kg, and 20 mg/kg doses of CM398 resulted in p values < 0.001 . All of the above p values show statistical significance. While none of the doses produce locomotor activity resembling the vehicle, all of the doses for CM304 and for CM398 produce more locomotor activity than the 2.5 mg/kg (i.p.) CP 55,940 dose. Additionally, CM398 appears to have a dose-dependent sedative effect. This has been seen with other sigma-2 compounds and may be a behavior that is associated with sigma-2 receptors, but this requires further investigation.

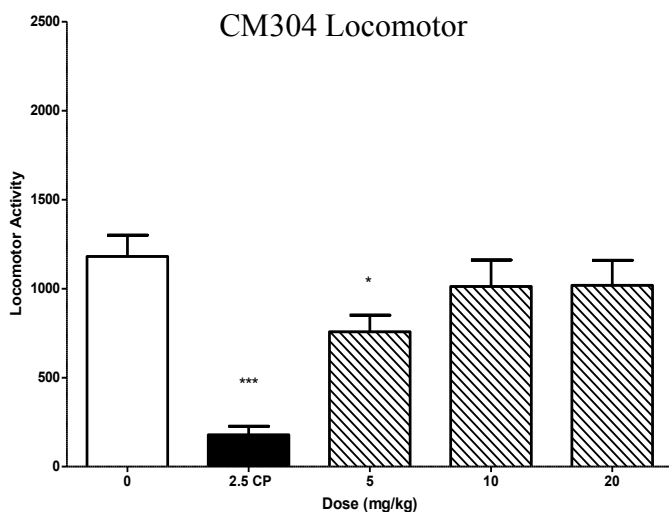


Figure 1

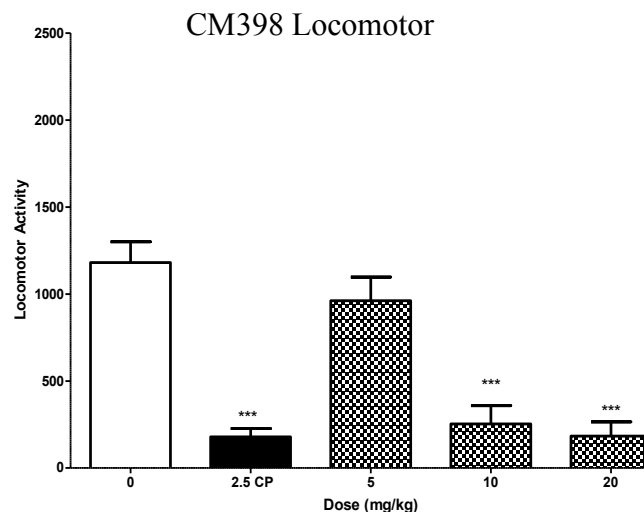


Figure 2

Figures 1-2: Locomotor increase post-CM304 i.p. injection (Figure 1) and post-CM398 i.p. injection (Figure 2) as compared to the vehicle and to CP55,940.

Figures 3 and 4 illustrate the locomotor activity response curves for morphine and CP 55,940, respectively. For morphine, as the dose increases, the locomotor activity increases. In fact, the highest dose of morphine (5 mg/kg) resulted in greater locomotor activity than the vehicle. This result is consistent with previous literature reports (Babbini et al., 1972). However, none of this data was statistically significant. On the other hand, the highest dose of CP 55,940 (0.5 mg/kg) produced the least locomotor activity. None of the CP 55,940 doses produced more activity than the vehicle. Although the doses did not produce more activity than the vehicle, the 0.1 mg/kg dose produced a p value < 0.05 and the 0.5 mg/kg dose produced a p value < 0.01 , both illustrating statistical significance.

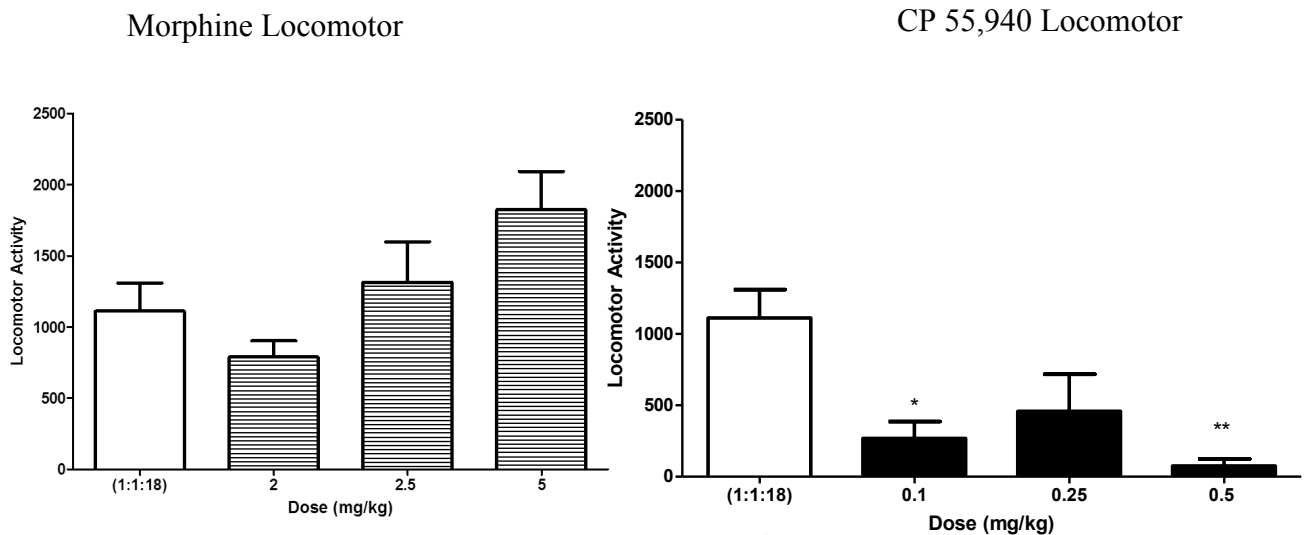


Figure 3

Figure 4

Figures 3-4: Locomotor activity post-morphine i.p. injection (Figure 3) and post-CP 55,940 (Figure 4) as compared to the vehicle.

II. Hotplate Assay

Figures 5-6 show the dose responses of CM304 (Figure 5) and CM398 (Figure 6) on perceived pain antinociception in the hotplate assay as compared to the vehicle and the 2.5 mg/kg dose of CP 55,940. Figure 5 demonstrates the fact that the dose of CM304 does not have a large effect on the perceived pain on the hotplate. Additionally, these doses produce results comparable to the results obtained from the vehicle administration. The only analysis showing statistical significance was 2.5 mg/kg dose of CP 55,940 with CM304 ($p < 0.001$). Figure 6 similarly demonstrates that the dose of CM398 does not have a statistically significant effect on the hotplate assay and produces results comparable to the vehicle.

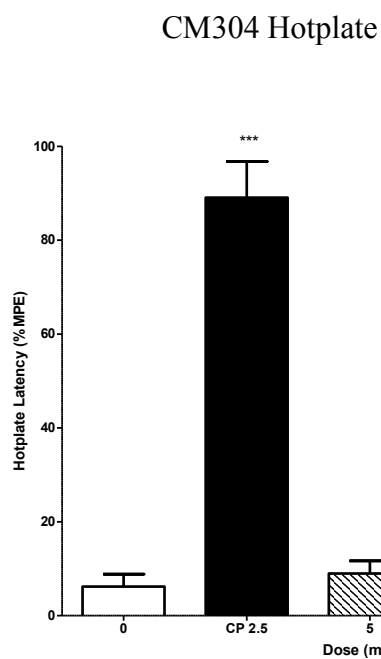


Figure 5

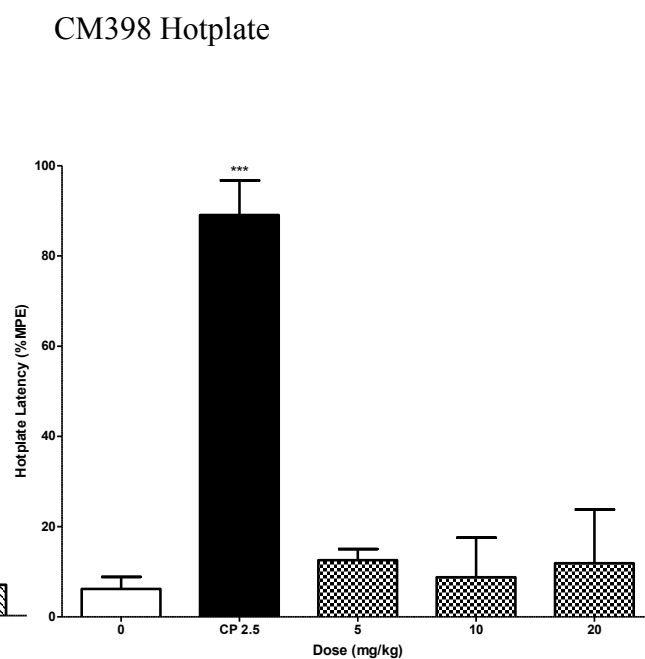


Figure 6

Figures 5-6: Different doses of CM304 and CM398 are injected i.p. to evaluate perceived antinociceptive pain in the hotplate assay and compared to the vehicle and a 2.5 mg/kg dose of CP 55,940.

Figures 7-8 show the dose response of morphine (Figure 7) and CP 55,940 (Figure 8) on perceived pain antinociception in the hotplate assay as compared to the vehicle. Figure 7 illustrates that the highest dose of morphine produced the largest hotplate latency. Additionally, the highest dose (5 mg/kg) was the only dose with statistically significant results ($p < 0.001$). On the other hand, Figure 8 shows the typical response: as the dose of CP 55,940 increases, the hotplate latency also increases. All of the doses of CP 55,940 showed p values < 0.001 . Similar to morphine, the highest dose of CP 55,940 produced the largest effect of perceived pain. All of the doses for morphine and CP 55,940 resulted in diminished pain perception. This assay, in addition to the tail-flick assay, made it evident which doses should be used for the potentiation studies. 2mg/kg morphine and 0.1 mg/kg CP 55,940 were chosen because they produced some changes in hotplate and tail-flick latencies but no results that were significantly different.

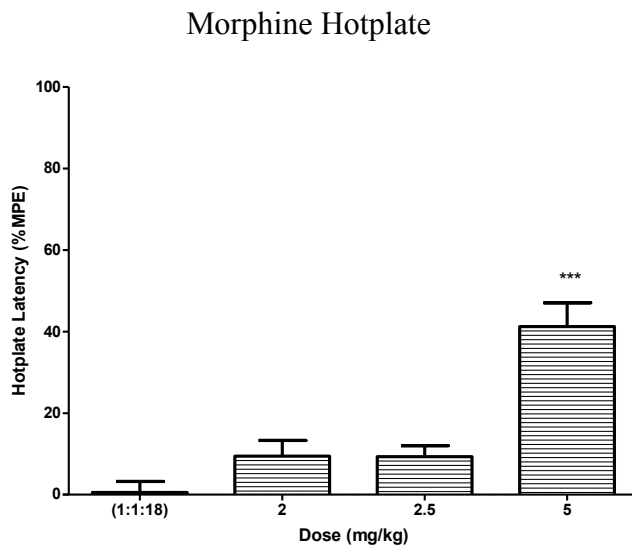


Figure 7

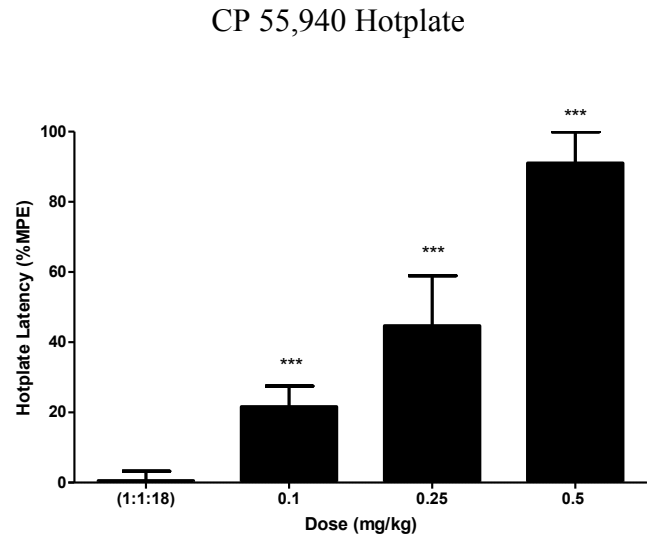


Figure 8

Figures 7-8: Different doses of morphine and CP 55,940 are injected i.p. to evaluate perceived antinociceptive pain in the hotplate assay and compared to the vehicle.

III. Catalepsy Assay

All doses of both CM304 (Figure 9) and CM398 (Figure 10) failed to create a major cataleptic effects thirty minutes post-injection. Figures 9 and 10 also reveal that a 2.5mg/kg dose of CP 55,940 creates a large cataleptic effect, much larger than any of the CM304 or CM398 doses. Both figures show that the 2.5 mg/kg dose CP 55,940, and no sigma antagonists, produced statistically significant results ($p < 0.001$).

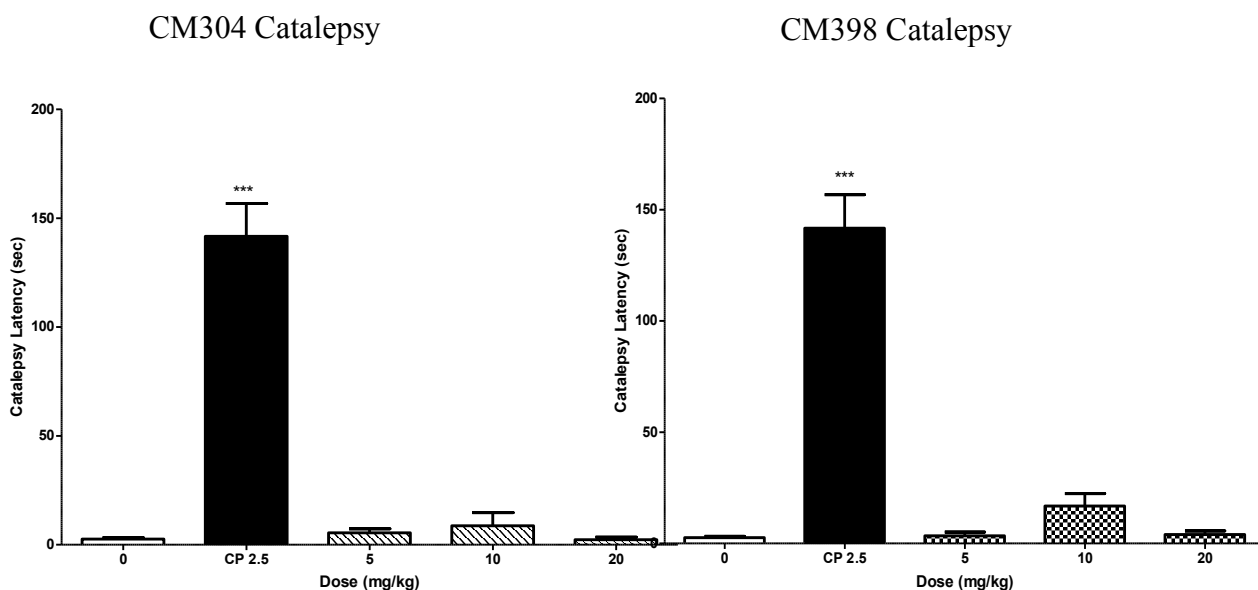


Figure 9

Figure 10

Figures 9-10 illustrate the lack of psychoactive effects due to CM304 (Figure 9) and CM398 (Figure 10), as compared to 2.5mg/kg CP 55,940.

In Figure 11, it is noteworthy that none of the doses of morphine produced statistically significant cataleptic effects, showing little to no psychoactive effects of the drug. All of the morphine doses produce results comparable to the vehicle. Figure 12 shows that as the dose of CP 55,940 increases, the catalepsy latency also increases. The highest dose of

CP 55,940 produced the largest effects and the lowest dose produced results comparable to the vehicle. The analysis of the highest dose of CP 55,940 revealed a p value <0.01.

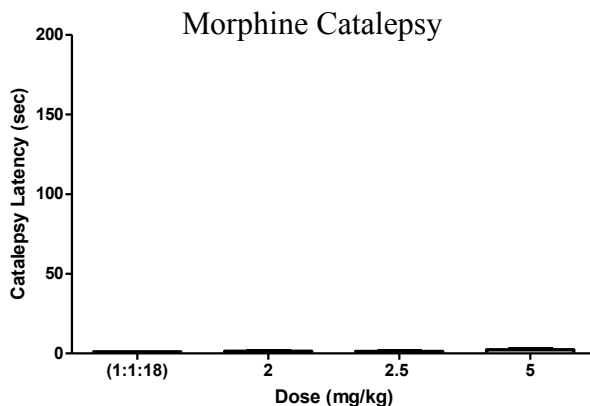


Figure 11

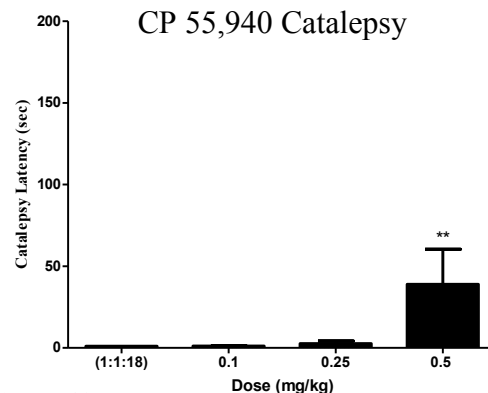


Figure 12

Figures 11-12 show the psychoactive effects of morphine and CP 55,940, respectively, compared to the vehicle.

IV. Body Temperature

Both CM304 (Figure 13) and CM398 (Figure 14) showed a decrease in core body temperature at all doses. For CM304 the greatest decrease was with a 10mg/kg dose, while CM398 had the greatest decrease at a 20mg/kg dose. None of the doses for either CM304 or CM398 approached the change in body temperature for 2.5mg/kg CP 55,940. Both figures show $p < 0.001$ for 2.5 mg/kg dose CP 55,940. Figure 14 shows statistical significance for both 10 mg/kg and 20 mg/kg doses of CM398 ($p < 0.001$).

CM304 Body Temperature

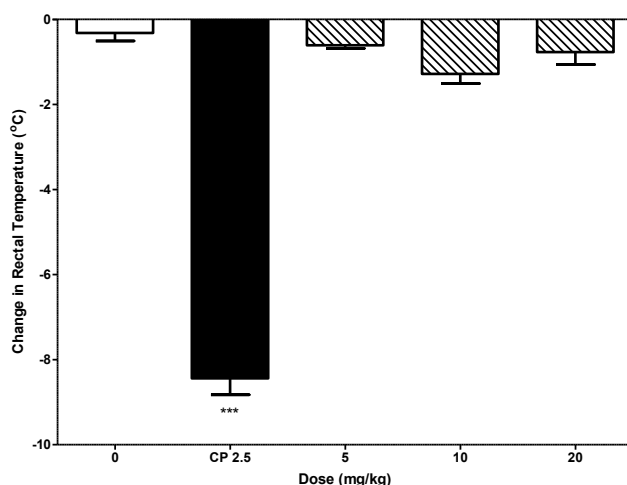


Figure 13

CM398 Body Temperature

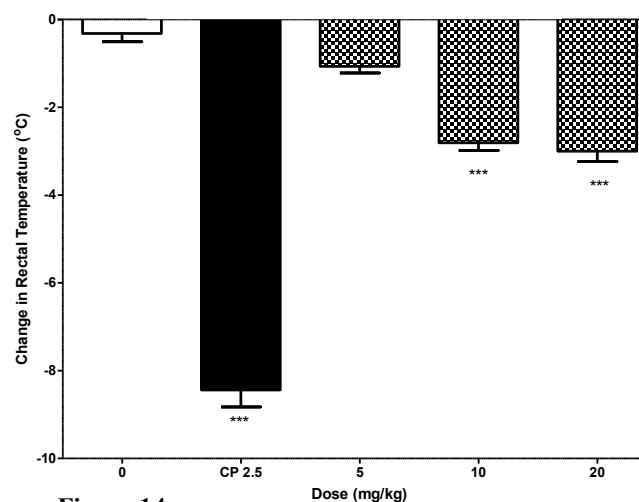


Figure 14

Figures 13 and 14 show the reduction of core body temperature 30 minutes post-injection of CM304 (Figure 13) and CM398 (Figure 14), compared to 2.5mg/kg CP 55,940.

Morphine (Figure 15) shows both increases and decreases in core body temperature at different doses. Regardless if there was an increase or a decrease, the absolute value of the change is very small and not statistically significant. Figure 16 also shows both increases and decreases at different doses of CP 55,940. The greatest change is produced by the highest dose of CP 55,940. This is also the only result that was statistically significant ($p < 0.01$). In both figures, the vehicle produces a very slight decrease in core body temperature.

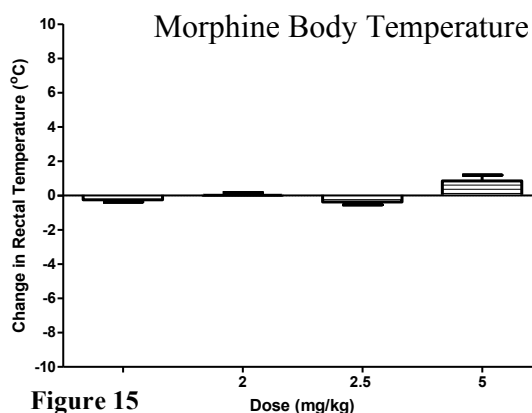


Figure 15

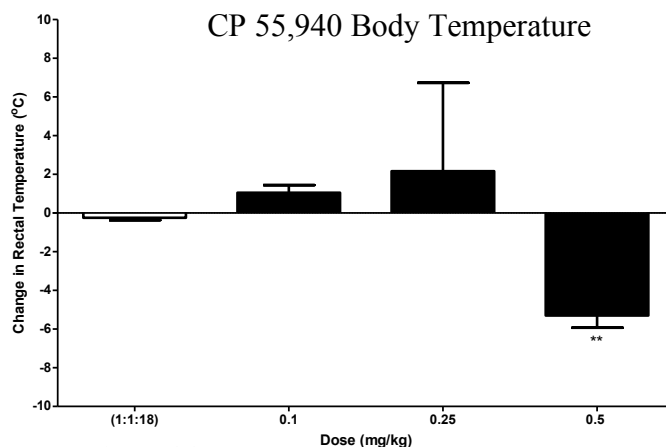
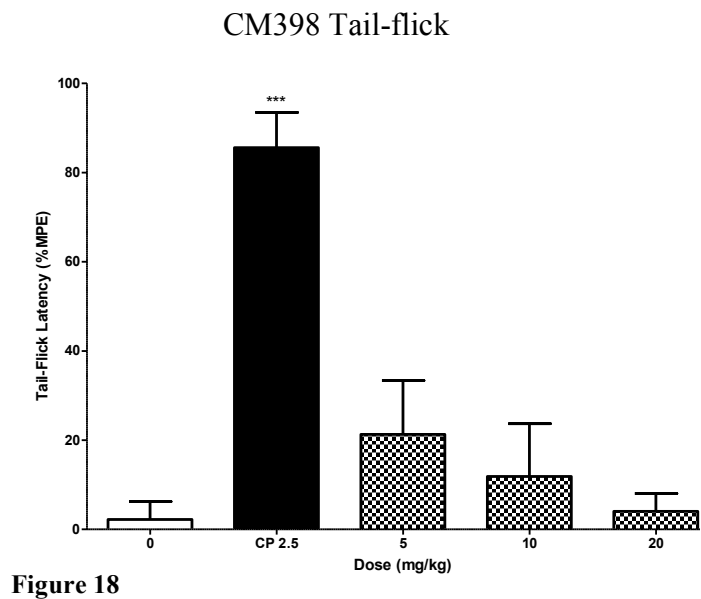
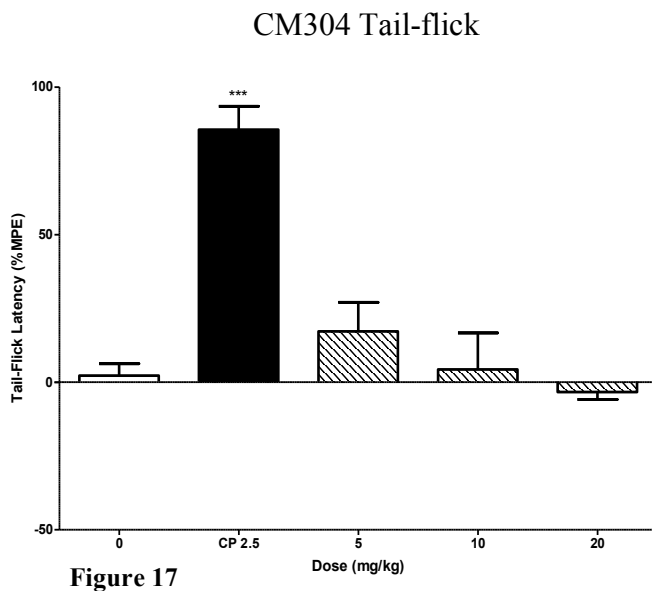


Figure 16

Figures 15-16 show both increases and decreases of core body temperature when administered with morphine (Figure 15) or CP 55,940 (Figure 16) 30 minutes post-injection, as compared to the vehicle.

V. Tail-flick Assay

CM304 (Figure 17) produced the most spinal antinociceptive actions in the tail-flick assay at the lowest dose. As the dose increased, the tail-flick latency also decreased. However, none of the effects from CM304 were statistically significant. Similarly, Figure 18 shows that as the dose of CM398 increases, the spinal antinociceptive actions decrease, but again, these results are not statistically significant. In both graphs, 2.5mg/kg CP 55,940 shows the largest tail-flick latency and the only statistically significant values ($p < 0.01$ for both).



Figures 17-18: The spinal antinociceptive actions in the tail-flick assay evaluated at 30 minutes post-injection of CM304 (Figure 17) and CM398 (Figure 18), and compared to CP 55,940.

Morphine (Figure 19) produces increasing tail-flick latency with an increasing dose. The result at all of the doses are much larger than the results of the administration of the vehicle. Both 5 mg/kg and 2.5 mg/kg doses produced p values < 0.01 . Similarly, CP

55,940 (Figure 20) shows that as the dose of the drug increases, the tail-flick latency also increases. Again, all of the doses produce greater results than the vehicle. The 2mg/kg dose of morphine and 0.1mg/kg dose of CP 55,940 produced ideal results in this assay (in addition to the hotplate assay) to be used for the potentiation studies. Figure 20 also shows that the 0.5 mg/kg dose of CP 55,940 generated a p value < 0.001.

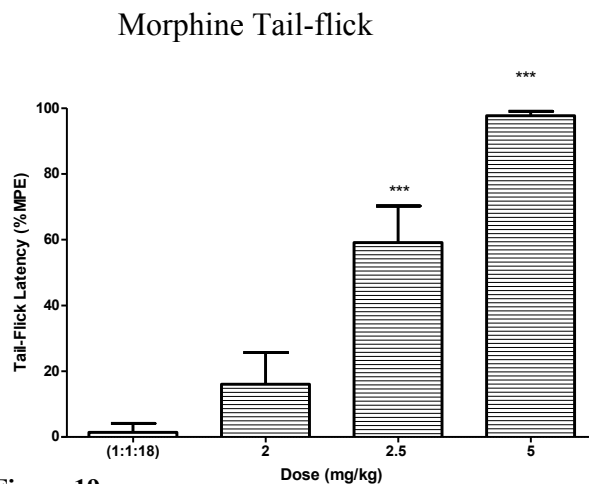


Figure 19

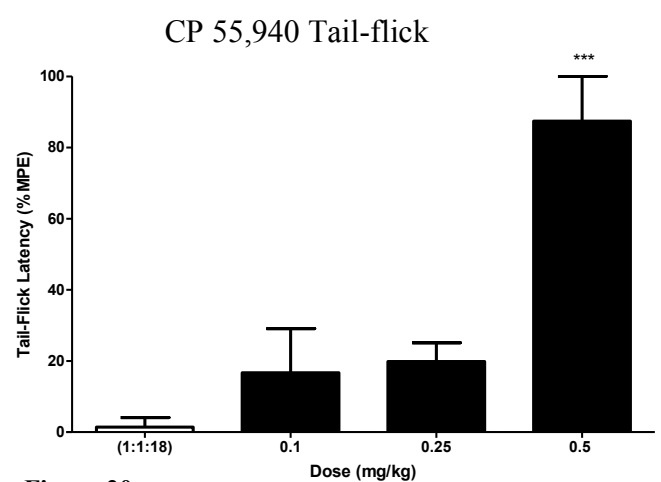


Figure 20

Figures 19-20: Tail-flick latencies 30 minutes post-injection for morphine (Figure 19) and for CP 55,940 (Figure 20), as compared to the vehicle.

Potentiation Studies

I. Hotplate Assay

The left side of Figure 21 shows the effects of the administration of one single drug on perceived pain on the hotplate. The right side of the graph shows the potentiation studies: the administration of an antagonist drug against a challenge drug. The graph shows that a 2 mg/kg dose of morphine was potentiated when administered in conjunction with a sigma-1 antagonist (CM304). Additionally, a 0.1 mg/kg dose of CP 55,940 was potentiated by both AZ66 (20 mg/kg) [p<0.05], a general sigma

antagonist, and two different doses of CM304 (20, 45 mg/kg [$p < 0.01$]). CM304 was able to enhance the perceived pain analgesic effects of both morphine and CP 55,940.

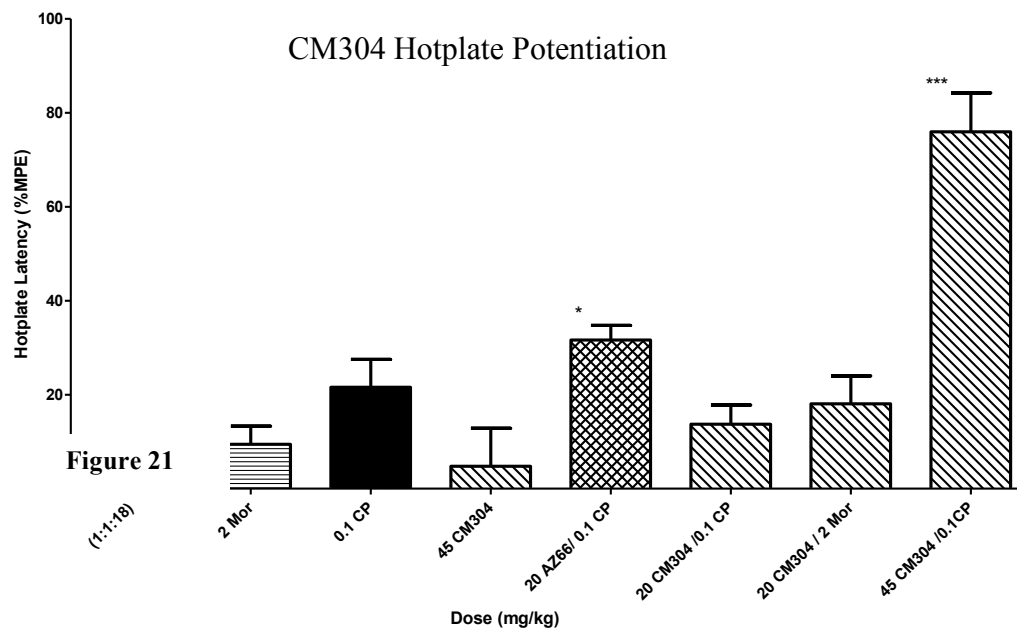


Figure 21: The potentiation effects of CM304 on hotplate latency are demonstrated for both morphine and CP 55,940.

Similar to Figure 21, the left side of Figure 22 shows the hotplate latencies for the administration of a single drug (the challenge drugs). The right side illustrates giving an antagonist prior to giving the challenge drug. Both doses of CM398 (20, 45 mg/kg), a sigma-2 antagonist, failed to potentiate the perceived pain analgesic effects of CP 55,940. On the other hand, CM398 was capable of potentiating the effects of 2mg/kg morphine. Lastly, 0.1 CP 55,940 was capable of being potentiated by 20 mg/kg of AZ66 ($p < 0.05$).

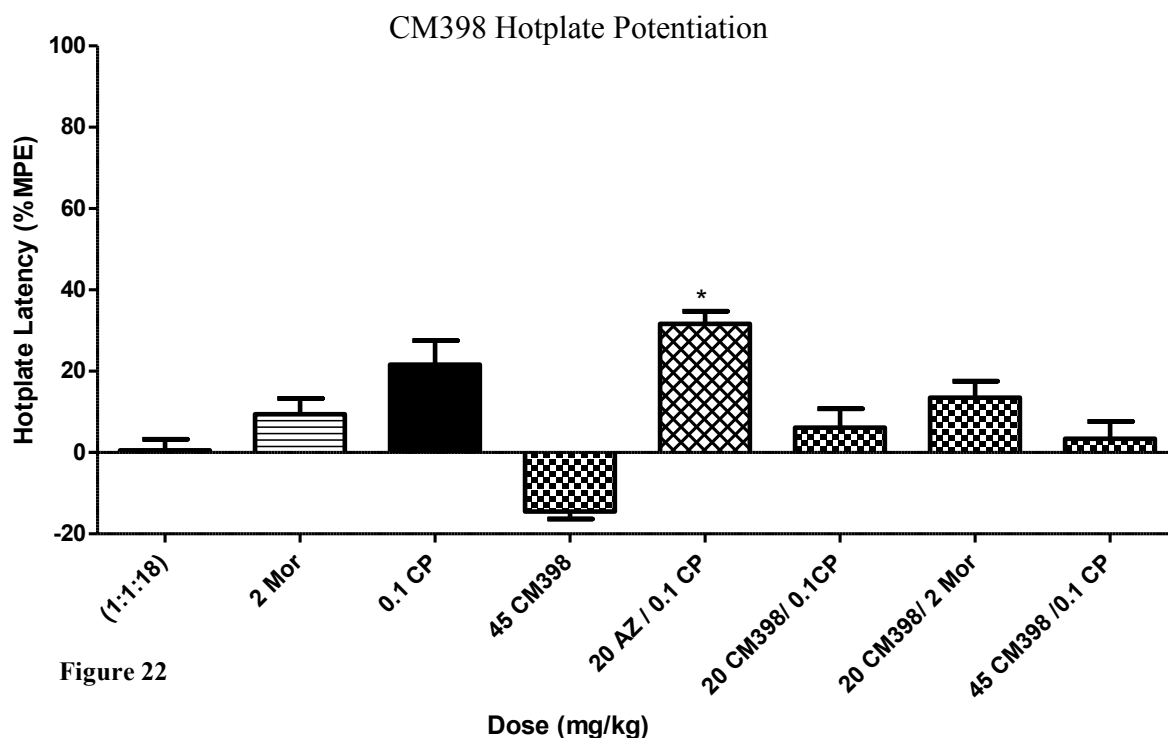


Figure 22

Figure 22: Perceived pain analgesic potentiation observed with CM398, a sigma-2 antagonist, and AZ66, a general sigma antagonist.

II. Tail-flick Assay

Figure 23 illustrates the potentiation effects of CM304 and AZ66 in conjunction with morphine and CP 55,940 on spinal analgesia with respect to the tail-flick. 2 mg/kg dose of morphine failed to be potentiated by 20 mg/kg CM304. Additionally, CM304 failed to potentiate the effects of 0.1mg/kg CP 55,940. However when the dose of CM304 was increased to 45 mg/kg, the tail-flick latency of 0.1 CP 55,940 was enhanced ($p < 0.05$). Lastly, AZ66 potentiated the effects of the 0.1mg/kg CP dose ($p < 0.01$).

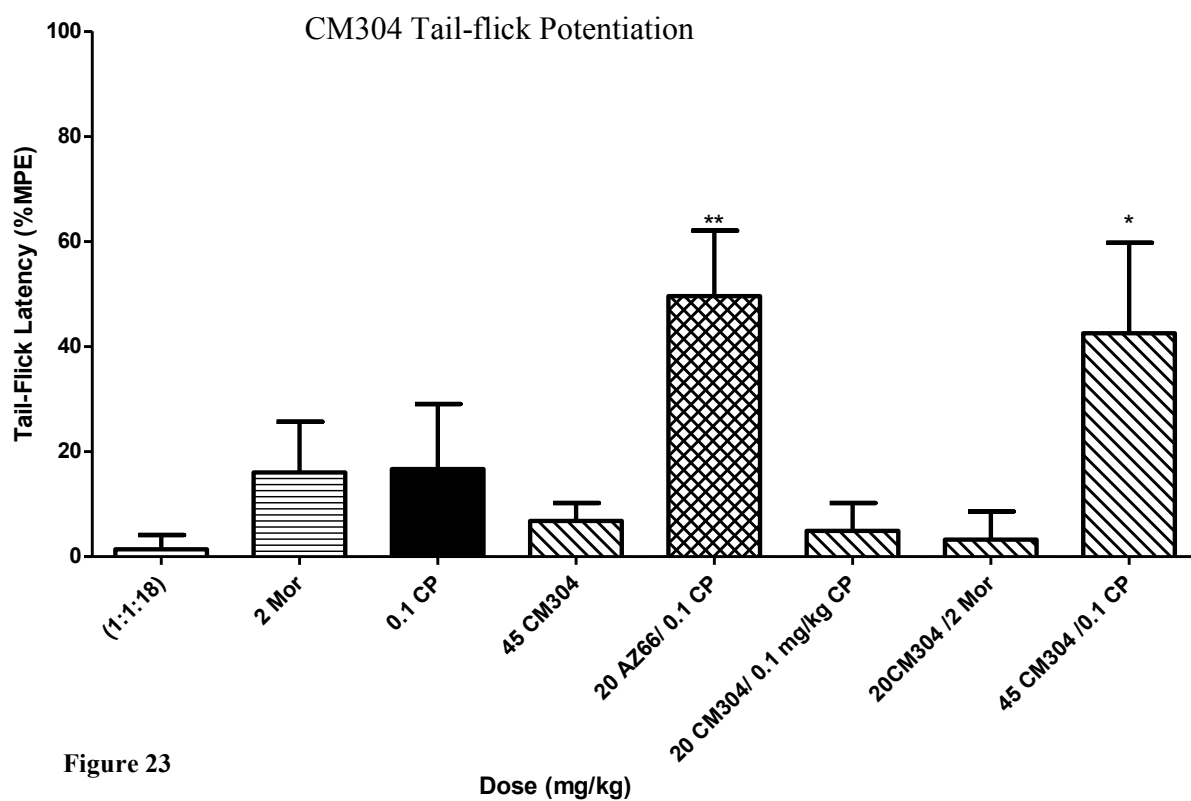


Figure 23

Figure 23: The potentiation effects of CM398 and AZ66 on spinal antinociception are illustrated.

In Figure 24 CM398, a sigma-2 antagonist, failed to potentiate the spinal analgesic effects of both morphine and CP 55,940, regardless of its dose (20, 45 mg/kg). 20 mg/kg AZ66, however, was able to potentiate the effects of 0.1 CP 55,940 ($p < 0.01$).

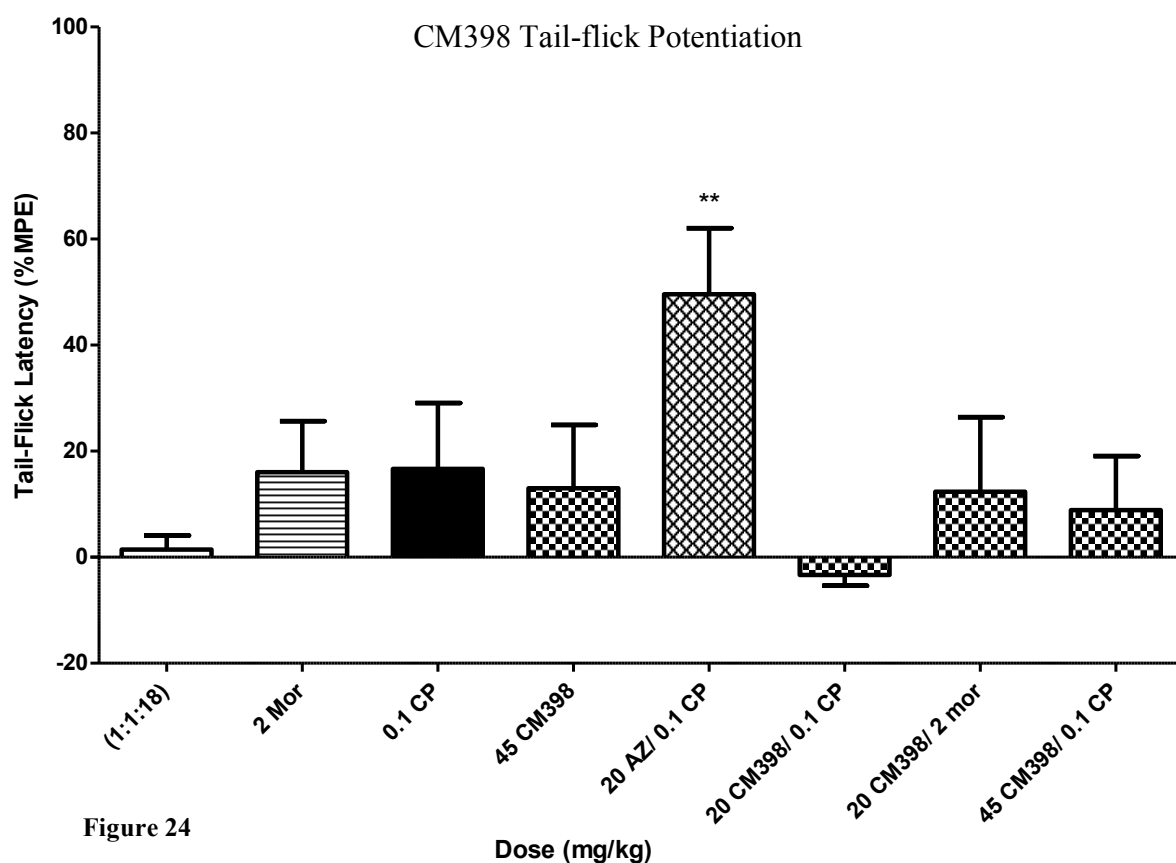


Figure 24: Spinal analgesic effects are not potentiated by different doses of CM398, a sigma-2 antagonist.

IV. Discussion

CM304 and CM398 are sigma-1 and sigma-2 antagonists, respectively. After the effects of each of these drugs were evaluated via the tetrad assay, it was determined that these drugs, on their own, do not yield significant psychoactive, central analgesia, peripheral analgesia, sedative, or hypothermic effects. This fact can also be confirmed by previous literature that demonstrated that sigma antagonists do not produce these effects without being co-administered with another drug (Zeng et al., 2014). Additionally, the well-known effects of morphine were confirmed in our tetrad assay: morphine showed little sedative effects, increased perceived pain analgesia, few psychoactive effects, a slight change in body temperature, and an increase in spinal antinociception. Lastly, CP 55,940

brought about the expected high psychoactive and high sedative effects along with an increase in central and peripheral analgesia (Melvin et al., 1993).

The 20 mg/kg dose of CM304 was able to potentiate the effects of morphine but not the effects of CP 55,940 on the hotplate assay. Once the dose was increased to 45 mg/kg, CM304 could enhance the perceived pain analgesia. On the other hand, both 20mg/kg and 45 mg/kg of CM398 attenuated supraspinal analgesia. When examining the antagonists' effects on spinal analgesia, 20 mg/kg CM304 attenuated the effects of both CP 55,940 and morphine. Once the dose was increased to 45 mg/kg, CM304 was able to potentiate the effects of only CP 55,940. Dissimilarly, both doses of CM398 were incapable of potentiating either morphine or CP 55,940. The general sigma antagonist, AZ66, potentiated the effects of CP 55,940 for both the hotplate and the tail-flick assays.

These results demonstrate that administering a sigma-1 antagonist, as opposed to a sigma-2 antagonist, in conjunction with either an opiate or a cannabinoid will potentiate the effects of the challenge drug. This can serve as a potentially very important part of future pain management research. Knowing that a sigma-1 antagonist instead of a sigma-2 antagonist can potentiate the perceived pain analgesic effects of an opiate and a cannabinoid can allow scientists to administer a more specific drug, providing researchers with a greater understanding of the mechanism.

V. Conclusion

For the first time, an interaction has been shown between the sigma receptors and the endocannabinoid system. This opens a multitude of new areas of research. Beyond the analgesic development that has begun here, research will be conducted to understand what other behaviors may be modulated through sigma receptors. Additionally, the major question that these results pose is whether or not the ability of sigma-1 to

potentiate the analgesic effects of the drug will also potentiate the psychoactive behavior associated with cannabinoids. The interplay of these additional behaviors with the endocannabinoid system can also be evaluated in future research. This research lays important groundwork for the research of the future.

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